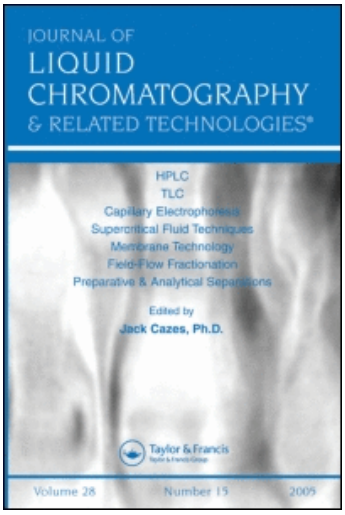

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A VALIDATED HPLC METHOD FOR SEPARATION AND DETERMINATION OF EPINASTINE HYDROCHLORIDE ENANTIOMERS

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□ *A simple, rapid, and validated method for separation and determination of epinastine hydrochloride was developed. Epinastine hydrochloride enantiomers was separated and determined on a Chiralcel® OD-R column (250 × 4.6 mm i.d., 0.5 μm particle size), using a mixture of n-hexane: isopropanol: diethylamine: triflouroacetic acid (85: 15: 0.1: 0.1% v/v/v/v) as a mobile phase at 20°C and at a flow rate of 1 mL/min. The UV detector was set to 254 nm. Epinastine hydrochloride 1000 μg/mL was used as an external standard. The applied HPLC method allowed the separation and quantification of epinastine hydrochloride enantiomers with good linearity ($r > 0.999$) in the studied range. The relative standard deviations (RSD) were 1.076 and 0.769% for the epinastine hydrochloride enantiomers with accuracy of 99.65 and 99.77 for the enantiomeric pair separated. The limit of detection and limit of quantification of epinastine hydrochloride enantiomers were found to be 20 and 60 μg/mL, respectively. The method was validated through the parameters of linearity, accuracy, precision, and robustness. The HPLC method was applied for the quantitative determination of epinastine hydrochloride in pharmaceutical formulations.*

Keywords chiral analysis, Chiralcel® OD-R, chiral separation, column liquid chromatography, drug analysis, epinastine, tris (3,5-dimethylphenyl carbamate) cellulose

INTRODUCTION

Epinastine (EPN), (±) -3-amino-9, 13b-dihydro-1H-dibenz[c,f] imidazo [1,5-a] azepine,[1,2] Figure 1, is a newly developed, non-sedating, histamine H_1 antagonist that is used as an anti-allergic agent and for the treatment of asthma. EPN is a clinically valuable agent, as it possesses weak potency on the central nervous system (CNS) because of its low rate of entry into the

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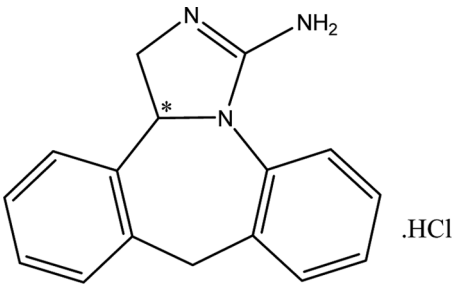

FIGURE 1 Chemical structure of Epinastine hydrochloride. Astrik (*) denotes the position of chiral centre.

CNS.[2–4] On the other hand, EPN ophthalmic solutions are applied to prevent itching of the eyes caused by allergic conjunctivitis.[5]

This drug was reported to be mainly excreted into urine and faeces in an unchanged form. Therefore, the kinetics of EPN may be relatively more resistant to drug induced metabolic inhibition or hepatic dysfunction compared with other conventional non-sedating antihistamines, such as terfenadine or astemizole, whose metabolism is highly altered by metabolic inhibition or hepatic dysfunction.[6–9]

These pharmacokinetic properties of EPN make it a potential replacement for conventional non-sedating àntihistamines; it is of great clinical relevance that its precise pharmacokinetic properties are investigated under several clinical states, such as hepatic or renal dysfunction. Thus, an analytical method for the determination of EPN is of urgent concern.

Ogiso et al.,[10] in their report focused on a preclinical phase I study and referred to a procedure for the determination of EPN. However, their procedure is lacking in some important aspects. They did not employ an internal standard as an analytical carrier and they did not carry out assay validation. Moreover, their method is complicated because it requires fluorescent labeling. Ohtani et al.[9] described a high performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection for the quantitative determination of EPN in plasma. Vera-Candioti et al.[11] used a capillary electrophoretic method for the determination of EPN in human serum; multiple response criteria were successfully used to optimize the separation of two analytes: EPN and lidocaine hydrochloride, which is used as an internal standard. Leonov and Bielory had reviewed the role of chirality of several ocular agents including EPN.[12] Tasaka et al.[13] found no significant difference between L- and D- enantiomers of epinastine in the antihistaminic and central nervous system depression effects. On the other hand, Nishi et al.[14] tried the separation of epinastine enantiomers by capillary electrophoresis and HPLC utilizing crown ethers chiral selectors. Although epinastine enantiomers were successfully resolved by capillary

electrophoresis, yet the adopted HPLC method using the Crownpak CR (+) column could not separate epinastine enantiomers.[14]

The objective of this work is to develop a simple, rapid, and validated method for separation and determination of epinastine hydrochloride enantiomers on a Chiralcel® OD-R column (250 × 4.6 mm i.d., 0.5 μm particle sizes) and its application to the analysis of the drug in pharmaceutical eye drops formulation.

EXPERIMENTAL

Chemicals

Epinastine hydrochloride reference standard was obtained from Nippon Boehringer Ingelheim (Hyôgo, Japan).

n-Hexane, isopropanol, diethylamine, and trifluoroacetic acid (HPLC grade) were obtained from Merck (Darmstadt, Germany). Sodium hydroxide, anhydrous sodium sulphate, and ethyl acetate of analytical grades were purchased from Sigma Chemicals (St. Louis, Mo, USA). Pharmaceutical preparation Relestat® eye drops labeled to contain 0.5 mg/mL epinastine hydrochloride, were manufactured by Allergen, West Port, Co. Mayo, Ireland, Batch. No. E52170.

Instrumentation and Analytical Conditions

The HPLC unit was an Agilent 1100 series apparatus equipped with a quaternary pump, a vacuum degasser, a column oven, a diode array UV detector, and HP chemstation software. The column used was Chiralcel® OD-R column (250 × 4.6 mm i.d., 0.5 μm particle size), Diacel Chemical Industries Ltd, France. The mobile phase consisted of n-hexane: isopropanol: diethylamine: trifluoroacetic acid (85:15:0.1:0.1% v/v/v/v). The flow rate was 1 mL/min. All the samples were measured at a wavelength of 254 nm and at 20°C.

Preparation of the Standard Solutions

Epinastine hydrochloride reference standard (10 mg) was neutralized with 1 M NaOH, extracted with ethyl acetate (10 mL × 3). The combined organic layers were washed with distilled water, separated, dried (Na_2SO_4 anhydrous), and evaporated under reduced pressure to dryness. The residue was dissolved in isopropanol (10 mL) to achieve a concentration of 1 mg/mL. This solution was freshly prepared on a daily basis.

Determination of Epinastine Hydrochloride Enantiomers

For construction of the calibration graphs, aliquots (1–10 mL) of 1 mg/mL epinastine standard solution was transferred into a series of 10 mL measuring flasks and completed to volume with isopropanol. An aliquot of 20 µL of the solution from each flask was injected into the HPLC at a flow rate at 1 mL/minute and at wavelength 254 nm, using 20 µL equivalents to 1 mg/mL of epinastine as an external standard. The ratio of peak area corresponding to the concentration of each sample was measured and a calibration graph representing the relation between concentration and ratio of peak area was constructed. Concentration of unknown samples could be derived from the calibration graph or calculated from the following regression equation.

Enantiomer 1:

$$Y = 0.001x + 0.0066 \quad r = 0.9997$$

Enantiomer 2:

$$Y = 0.001x + 0.0018 \quad r = 0.9999$$

Where, Y = peak area of sample/peak area of external standard

x = concentration of epinastine in µg/mL

r = correlationcoefficient

During the chromatographic analysis the following parameters were measured:

k_1 and k_2: Capacity factors of the first and second eluted enantiomers and were 2.27 and 3.47, respectively.

α: Selectivity factor, $\alpha = k_2/k_1 = 1.53$.

R_s: Resolution factor was found to be 6.60, calculated according to the following equation, $R_s = 2(t_2 - t_1)/w_1 + w_2$.

Where, w_1 and w_2 are the base line band width obtained by drawing tangents to the inflexion points of the chromatographic peaks for eantiomer **1** and enatiomer **2**, respectively, and t_2 and t_1 are the retention times of the second and first eluted enantiomer, respectively.

Determination of Epinastine Hydrochloride Enantiomers in Relestat® Eye Drops

Four bottles of Relestat eye drops containing 10 mg epinastine hydrochloride were neutralized with NaOH (1 M), extracted with ethyl acetate

(10 mL × 3). The combined organic layers were washed with distilled water, separated, dried (Na_2SO_4 anhydrous), and evaporated under reduced pressure to dryness. The residue was dissolved in isopropanol (10 mL). Epinastine concentration was determined by taking (1–10 mL) and completed to a volume of 10 mL with isopropanol, if necessary, and proceeding as previously described.

METHOD VALIDATION

The methods were validated according to the International Conference of Harmonization guidelines (ICH – Q2B 1996), for validation of analytical procedures.[15]

Linearity

The calibration curve was obtained with seven concentrations of the standard solution 100–1000 μg/mL. The solutions were prepared in triplicate. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method.

Precision

The precision of the assay was determined by repeatability (intra-day) and intermediate precision (inter-day). Intra-day precision was evaluated by assaying the sample at the same concentration and during the same day. Six sample solutions 500 μg/mL were prepared and assayed. The intermediate precision (inter-day) was studied by comparing the assays on different days (3 days).

Accuracy

The accuracy of an analytical method is determined by how close the test results obtained by that method come to the true value. It can be determined by application of the analytical procedure to an analytical of known purity (for the drug substance) or by recovery studies, where a known amount of the standard is spiked in the placebo (for drug product). In the present study, a number of different solutions were prepared with a known added amount of drug substance and injected in triplicate. Percent recoveries of response factor (area and concentration) were calculated, which indicated the accuracy of the proposed method.

Robustness

The robustness of the HPLC method was determined by analysis of the samples under a variety of conditions by making small change in the mobile phase composition at flow rates (0.8–1.2 mL/min), at temperature of the column (18–25°C), and by changing the wavelength (250–258 nm) with mean ± RSD (99.98 ± 0.85%).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) is defined as the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified, and the limit of quantification (LOQ) was defined as the lowest concentration of analyte in a sample that can be determined with acceptable precision and accuracy.

RESULTS AND DISCUSSION

The development of a stereoselective HPLC method for the determination of enantiomeric drugs has received considerable attention in recent years because of its importance in analysis of quality control of pharmaceutical formulations. In this study, an HPLC method for the separation and quantitation of epinastine hydrochloride enantiomers in its pharmaceutical formulations was developed.

The chromatographic conditions were adjusted in order to provide a reliable assay performance. Mobile phase selection was based on peak parameters, runtime, ease of preparation, and cost. A typical chromatogram is shown in Figure 2, for the analysis and separation of a sample solution of epinastine hydrochloride using 1000 μg/mL epinastine hydrochloride as an external standard.

The retention time was observed at 10.838 min for enantiomer **1** and at 14.802 min for enantiomer **2** using 1000 μg/mL of epinastine standard as an external standard.

The limit of detection (LOD) and limit of quantitation (LOQ) were obtained using the slope and standard deviation of the intercept from three curves and determined by the linear regression line, which were 20 and 60 μg/mL, respectively.

These values were also used in an experimental assay confirming the calculation. The calibration curves for epinastine hydrochloride were constructed by plotting concentrations (μg/mL) versus peak area and showed good linearity in the 100–1000 μg/mL range. The representative linear equations were $Y = 0.001x + 0.0066$ for enantiomer **1** and $Y = 0.001\ x + 0.0018$

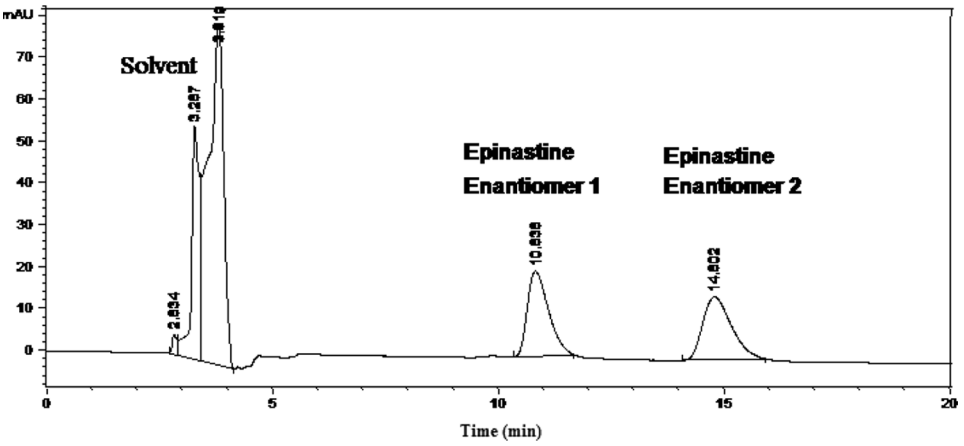

FIGURE 2 Chromatogram of epinastine Hydrochloride 500 µg/mL on Chiralcel® OD-R column (250 × 4.6 mm i.d., 0.5 µm particle size), Diacel Chemical Industries Ltd, France, using mixture of *n*-hexane: isopropanol: diethylamine: trifluoroacetic acid (85: 15: 0.1: 0.1% v/v/v/v) as a mobile phase at flow rate of 1 mL/min and wavelength of 254 nm at 20°C.

TABLE 1 Results of the Determination of Epinastine Hydrochloride in Relestate® Eye Drops by HPLC

Sample (µg)	Experimental Amount* (µg)	%	(RSD %)
Enantiomer **1**			
200.00	200.60	100.30	
	199.20	99.60	0.440
	200.80	100.40	
Enantiomer **2**			
200.00	200.70	100.35	
	199.50	99.75	0.361
	200.80	100.40	

*Mean of five determinations for each concentration.

TABLE 2 Determination of Authentic Epinastine Hydrochloride via the Suggested HPLC Method

Added Authentic µg/Ml	Found Authentic µg/mL for Enantiomer **1**	Recovery % of Enantiomer **1**	Found Authentic µg/mL of Enantiomer **2**	Recovery % of Enantiomer **2**
200	202.40	101.20	201.20	100.60
500	496.40	99.28	501.20	100.24
600	592.40	98.73	594.30	99.05
1000	994.00	99.40	991.90	99.19
Mean ± RSD*		99.65 ± 1.076		99.77 ± 0.769

*Average of at least three separate determinations.

TABLE 3 Statistical Comparison of the Results Obtained by Adopting the Proposed Method as Compared to the Reference Method* for Analysis of Epinastine Hydrochloride

Technique	Mean ± RSD*	N	Variance	Student (*t*.test)	F
Reference method	100.81 ± 0.542	6	0.298	–	–
HPLC for Enantiomer **1**	99.65 ± 1.076	4	1.149	2.086 (2.306)**	3.856 (5.410)**
HPLC for Enantiomer **2**	99.77 ± 0.769	4	0.588	2.035** (2.306)**	1.973 (5.410)**

*Quantitative UV spectrophotometry in methanol using A (1%, 1 cm) at 263 nm for determinations of epinastine hydrochloride.[16]

**The figures in parenthesis are the theoretical t and F values at ($p = 0.05$).

for enantiomer **2** with high correlations coefficients ($r = 0.9997$) and ($r = 0.9999$), respectively, where Y = peak area of sample/peak area of external standard

$$x = \text{concentration of epinastine in } \mu g/mL.$$

Accuracy and precision of the proposed method were assessed by performing triplicate analyses of the standard solutions. Three different concentrations, diluted with the mobile phase, were prepared in the linear range of the calibration curve and analyzed to determine intra-day variability and accuracy. The intra- and inter-day precision was calculated as the RSD%. The results and the mean values were 100.36 ± 0.56, 100.49 ± 0.86 for enantiomer **1** and 100.47 ± 0.63, 100.16 ± 0.90 for enantiomer **2**, respectively, which demonstrated good precision and accuracy.

When chromatographic conditions were intentionally altered, no significant effect was observed in the chromatogram, confirming the robustness of the method.

Results of the determination of epinastine in Relestat® eye drops formulations were shown in Table 1. The accuracy of the HPLC method for enantiomers **1** and **2** were 99.65% and 99.77%, respectively, confirming the accuracy of the proposed methods. The results were expressed in Table 2.

The proposed analytical method was compared with the reference method.[15] The calculated F-value for both enantiomers **1** and **2** ($F_{cal} = 3.856$) and ($F_{cal} = 1.973$), respectively, were found be less than the tabulated F-value ($F_{tab} = 5.410$) and ($F_{tab} = 5.410$) at a 1% significance level, respectively, Table 3.

CONCLUSION

The proposed HPLC method described a quantitative determination and separation of epinastine hydrochloride enantiomers in bulk drug and in pharmaceutical eye drops formulation. The validated HPLC method

is fast, precise, accurate, and efficient, and can be applied in routine analyses in quality control laboratories. The proposed method will be used to separate epinastine enantiomers for further pharmacological evaluation and study of their pharmacokinetic behavior, which is currently underway.

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